The Use of High Performance Liquid Chromatography for the Characterisation of the Unfolding and Aggregation of Dairy Proteins

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Running head: HPLC for dairy proteins

Revised and accepted in June 2019

Gaspard, S. J., & Brodkorb, A. (2019). The Use of High Performance Liquid Chromatography for the Characterization of the Unfolding and Aggregation of Dairy Proteins. In J. J. McManus (Ed.), *Protein Self-Assembly: Methods and Protocols* (pp. 103-115). New York, NY: Springer New York.

Abstract

High performance liquid chromatography (HPLC) is routinely used to identify and characterise proteins. HPLC can to understand protein aggregation processes in dairy products, which are induced by common industrial processing steps such as heat treatment. In this chapter, three complimentary chromatographic methods are described, which are based on the principles of size exclusion and reversed-phase chromatography. These methods are used to determine the degree of denaturation and aggregation of proteins, and estimate the molecular weight of these aggregates.

Key words: HPLC, proteins, denaturation, aggregation, reversed-phase chromatography, size exclusion chromatography

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1. Introduction

High performance liquid chromatography, abbreviated HPLC, is a routine technique developed in the 1960s to purify and analyse polar molecules with a high molecular weight in less than one hour **(*1*)**. Thanks to significant improvements in chromatography matrices and the packing of columns, HPLC can now be used as a tool to analyse peptides, proteins and biopolymers with great accuracy and reproducibility. The characterisation of proteins, in particular protein unfolding and aggregation, is of great importance in the field of biochemistry, but also widely used in food and biomaterial sciences. This chapter describes the use of HPLC in dairy chemistry, in particular for the characterisation of the state of dairy proteins (native, unfolded, aggregated) due to common process-induced changes during food production (e.g. heat treatment, high pressure, concentration, dehydration, change in acidity and ionic strength etc.). Chromatographic separation is based on the size (gel permeation or size exclusion chromatography, SEC-HPLC) or polarity (reversed-phase chromatography, RP-HPLC) of the protein material. By combining these methods, a detailed characterisation of the extent of protein denaturation and aggregation is possible **(*2-4*)**. The chromatography results contribute to the overall kinetic and structural understanding of heat-induced changes in the structure of dairy proteins, which is of high scientific and industrial interest. A summary of other methods for the quantification of dairy proteins can be found elsewhere **(*5*)**.

Three complimentary HPLC methods are described in this chapter:

Method 1: RP-HPLC method for the total quantification of dairy proteins (caseins and whey proteins, native and aggregated), based on a method by Visser *et al.* **(*6*)**. It allows the quantification of individual proteins, including those in aggregates. Sample treatment involves the disruption of the intermolecular disulphide bonds and non-covalent interactions by β-mercaptoethanol and urea **(*7*)**.

Method 2: RP-HPLC method for the quantification of native whey proteins based on a method by Beyer *et al.* **(*8,9*)**. Sample treatment involves the isoelectric precipitation and removal of denatured whey proteins. The degree of protein denaturation can be calculated from the difference between the

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44 total amount of proteins and that of the native proteins. This is only applicable for whey proteins,
45 which can unfold and denature because of their globular structure.

46 Method 3: SEC-HPLC method for the estimation of the molecular weight of the proteins and protein
47 aggregates. The method is suitable for molecular weight ranges between approximately 10^4 and
48 5×10^5 Da, depending on the choice of chromatography column.

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2. Materials

All solutions should be prepared using ultrapure water, such as Milli-Q® water and analytical or, if available, HPLC-grade reagents. All solutions containing acetonitrile (ACN) or trifluoroacetic acid (TFA) must be prepared in a fume hood using the correct PPE (lab coat, lab goggles and appropriate gloves).

A. *Preparation of Mobile Phase A (for method 1), 10% (v/v) acetonitrile (ACN) + 0.1% (v/v) trifluoroacetic acid (TFA) in water.* Carefully pour 200 mL of ACN into a 2 L volumetric flask. Add around 1,500 mL of water and 2 mL of TFA (*see* **Note 1**). Invert the solution to thoroughly mix the organic phase and water. Fill up to 2 L with water. Rinse a filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum filter the mobile phase with a 0.45 μm pore size hydrophilic filter (e.g. Durapore hydrophilic PVDF membrane filter type, Merck Millipore). Store at room temperature (*see* **Note 2**).

B. *Preparation of Mobile Phase B (for method 1 and 2), 90% (v/v) ACN + 0.1% (v/v) trifluoroacetic acid (TFA) in water.* First add 200 mL of water to a 2 L volumetric flask (*see* **Note 3**). Carefully add 2 mL of TFA and slowly add ACN up to 10 cm below the fill line. Invert the solution to mix thoroughly. Wait 20 min to fully equilibrate and fill up to the 2 L mark (*see* **Note 4**). Rinse a filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum filter the mobile phase with a 0.45 μm pore size hydrophobic filter (e.g Durapore hydrophobic PVDF membrane filter type, Merck Millipore). Store at room temperature (*see* **Note 2**).

C. *Preparation of Mobile Phase A (for method 2), 0.1% (v/v) trifluoroacetic acid (TFA) in water.* Mix approximately 1,800 mL of water and 2 mL of TFA (*see* **Note 1**). Make up to 2 L with water. Invert the solution to thoroughly mix the water and TFA. Rinse a filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum

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filter the mobile phase with a 0.45 μm pore size hydrophilic filter (e.g. Durapore hydrophilic PVDF membrane filter type, Merck Millipore). Store at room temperature (*see* **Note 5).**

D. *Preparation of the mobile phase (for method 3), 20 mM sodium phosphate, pH 7.0.* Prepare 1 L of 20 mM monobasic sodium phosphate (NaH_2PO_4) and 1 L of 20 mM dibasic sodium phosphate (Na_2HPO_4). Add solid sodium azide to reach a concentration of 0.05% (w/v) in both solutions to inhibit undesirable microbial growth. Add 900 mL of 20 mM dibasic sodium phosphate to a 2 L beaker and stir continuously. Slowly add 20 mM monobasic sodium phosphate until pH 7.0 is reached. Rinse a filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum filter the mobile phase through a 0.45 μm pore size hydrophilic filter (e.g. Durapore hydrophilic PVDF membrane filter type, Merck Millipore). Store at room temperature (*see* **Note 6**).

E. *0.1 M Sodium acetate/acetic acid buffer pH 4.6.* Prepare 0.1 M of sodium acetate with water in 500 mL volumetric flask, in the fume hood. Prepare 0.1 M acetic acid with water in a 500 mL volumetric flask, in the fume hood. Transfer 400 mL of 0.1 M acetic acid solution to a 1 L beaker and slowly add 0.1 M sodium acetate until pH 4.6 is reached. Store at room temperature (*see* **Note 7**).

F. *Denaturing sample buffer: 7 M urea + 20 mM bis-tris propane, pH 7.5.* Weigh 42 g of urea (M_w = 60.06 g/mol) and 0.56 g of bis-tris propane (1,3-bispropane, M_w = 282.334 g/mol) in a glass beaker with 80 mL of water (*see* **Note 8**). Stir and heat gently to aid dissolution. Adjust the pH to 7.5 using 0.1 M HCl or NaOH. Transfer to a 100 mL volumetric flask and rinse the transfer funnel with a small amount of water. Add water to 100 mL. Invert several times to mix thoroughly. (*see* **Note 9**).

G. *Molecular weight standards.* The molecular weight standards (*see* **Table 1**) are prepared in water.

(Table 1 near here)

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3. Methods

3.1 Method 1: Quantification of dairy proteins using RP-HPLC

3.1.1 Sample preparation for dairy protein quantification

Caseins exist in milk as large, colloidal particles (casein micelles, mean diameter ≈ 150 nm) suspended in the aqueous milk serum, the latter containing whey proteins. Caseins associate via non-covalent interactions **(*10*)**. In contrast to this, native whey proteins are in monomeric or dimeric form. Upon heating, whey proteins and caseins can associate via covalent disulphide bonds and other non-covalent interactions. For chromatographic separation, the proteins need to be dissociated and fully denatured prior injection onto the column. The non-covalent interactions and disulphide bonds can be disrupted by pre-treating samples with urea and β-mercaptoethanol. In this method, the samples are mixed with the denaturing sample buffer in a ratio of 1:20 (*see* **Note 10**).

1. In order to reach the desired final concentration of 0.2% (w/v) of proteins (*see* **Note 11**), standardise the protein sample to 3.5-4% (w/v) protein. The protein standards, native whey proteins and caseins are prepared in water.
2. Transfer the volume of sample buffer needed for a volume ratio sample:buffer of 1:20, to a polypropylene tube and add 50 μL of β-mercaptoethanol for every 10 mL of sample buffer.
3. Add 200 μL of each sample to 3.8 mL of urea and β-mercaptoethanol mixture. Vortex the samples. Leave at room temperature for 1 h and invert every 15 min (*see* **Note 12**).
4. Filter the samples through a 0.22 μm low protein binding and hydrophilic syringe filter (e.g. PVDF membrane filter type) into the HPLC vials. Fill to the neck.

3.1.2 HPLC system

The method requires a HPLC separation module with a UV/visible detector and the corresponding software for data analysis.

The results were obtained here using a Poroshell 300SB-C18 column measuring 2.1×75 mm from Agilent (Santa Clara, CA, USA).

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One chromatographic run takes 35 min per sample at a flow rate of 0.5 mL/min. The injection volume is 5 μL. The column temperature is set at 35°C.

3.1.3 HPLC run and analysis of the elution profiles

1. Equilibrate the column with 2 to 5 column volumes of a mobile phase mixture of 74% Mobile Phase A and 26% of mobile phase B at a flow rate of 0.5 mL/min. The absorbance is recorded at 214 and 280 nm (see **Note 13**). After equilibration, the absorbance should be constant and changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
2. Set up the HPLC instrument method to run the gradient detailed in **Table 2**, at a flow rate of 0.5 mL/min and at a column temperature of 35°C.
3. Inject 5 μl of a blank (water or mobile phase) at the beginning and end of each set of samples to verify a clean baseline. Inject 5 μL of the samples and the standards. The order for the injection should follow an increasing protein concentration to reduce the risk of cross-contamination.
4. Compare the elution time of the standards to the elution time of the unknown proteins to identify the peaks. Use the software functions to integrate the individual peaks and deduce the protein content of each protein from a calibration curve for each protein standard. Anticipated elution profiles of caseins and whey proteins at 214 nm are shown in **Figure 1**.

(Table 2 near here)

(Figure 1 near here)

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3.2 Method 2: Quantification of whey protein denaturation by RP-HPLC

3.2.1 Sample preparation

1. Dilute the protein standards in ultrapure water.

2. Dilute the protein samples in sodium acetate/acetic acid buffer at pH 4.6 to reach a protein concentration of 0.25% (w/v) (*see* **Note 11**). Separate the isoelectric precipitate by centrifugation at 14,000×g for 30 min at room temperature (*see* **Note 14**).

3. If dilution of the supernatant is necessary, dilute in a mixture of 80% Mobile Phase A and 20% of mobile phase B (*see* **Note 15**).

4. Discard the pellet and filter the supernatant and the protein standards through 0.45 μm low protein binding and hydrophilic syringe filter (e.g. PES membrane filter type, Sartorius, Göttingen, Germany) directly into the HPLC vials. Fill to the neck of the vial.

3.2.2 HPLC system

The method requires a HPLC separation module with a UV/visible detector and the corresponding software for data analysis.

The results were obtained using a C5 PolymerX RP1 column (*see* **Note 16**) measuring 150×4.6 mm from Phenomenex (Torrance, CA, USA). One chromatographic run takes 45 min per sample at a flow rate of 1 mL/min. The injection volume is 20 μL. The column temperature is set at 28°C.

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3.2.3 HPLC run and analysis of the elution profiles

1. Equilibrate the column with 2 to 5 column volumes of a mobile phase mixture of 80% Mobile Phase A and 20% of mobile phase B with a flow rate of 1 mL/min. The absorbance is recorded at 214 and 280 nm (*see* **Note 13**). After equilibration, the absorbance should be constant and changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
2. Set up the HPLC instrument method to run the gradient detailed in **Table 3**, at a flow rate of 1 mL/min and at a temperature of 28°C.
3. Inject 20 µl of a blank (water or mobile phase) at the beginning and end of each set of samples to verify a clean baseline. Inject 20 µl of the samples and the standards. The order for the injection should follow an increasing protein concentration to reduce the risk of cross-contamination.
4. Compare the elution time of the standards to the elution time of the unknown proteins to identify the peak. Use the software functions to integrate the individual peaks and deduce the protein content of each protein from a calibration curve for each protein standard. The amount of denatured protein is calculated as the difference between the initial amount of non-heated protein samples and the residual amount after heating, both determined by this method. Alternatively, the total (native + denatured) amount of protein can be determined by the method described in section 3.1.
5. Anticipated elution profiles of caseinomacropeptide (CMP), α-lactalbumin (α-la) and β-lactoglobulin (β-lg) at 280 nm and 214 nm are shown in **Figure 2**.

(Table 3 near here)

(Figure 2 near here)

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3.3 Method 3: Determination of the degree of protein aggregation by SEC-HPLC system

Two columns in series, TSK Gel G2000SW$_{XL}$ and TSK Gel G3000SW$_{XL}$ (Tosoh Bioscience GmbH, Griesheim, Germany) are used, preceded by a guard column to prevent potential column blockage. The dimensions of both columns are 7.8 x 300 mm (*see* **Note 17**).

The method requires a HPLC separation module, a UV/visible detector and the corresponding software for the elution analysis. The flow rate is 0.5 mL/min with an isocratic gradient of 20 mM sodium phosphate (pH 7.0). The total duration of the run is 60 min per sample. The injection volume is 20 µl. The column should remain at room temperature without the use of a column oven (*see* **Note 18**).

3.3.1 HPLC run and analysis of the elution profiles

The samples are standardised to 0.25% (w/v) protein in water (*see* **Note 11**) and filtered through 0.45 µm hydrophilic syringe filters with a low protein binding profile (e.g. PES membrane filter type, Sartorius, Göttingen, Germany). The molecular weight standards were prepared as described in section 2.

1. Equilibrate the column with 2 column volumes of 20 mM of sodium phosphate (pH 7.0; 0.05%, w/v, sodium azide) buffer at a flow rate of 0.5 mL/min. The absorbance is recorded at 214 and 280 nm (*see* **Note 13**). After equilibration, the absorbance at 280 nm should be constant and changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
2. Set up the HPLC instrument method to run an isocratic gradient of 20 mM sodium phosphate (pH 7.0) at a flow rate of 0.5 mL/min.
3. Inject 20 µl of a blank (water or mobile phase) at the beginning and end of each set of samples to verify a clean baseline. Then, inject 20 µl of the samples and the standards. The order for

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the injection should follow an increasing protein concentration to reduce the risk of cross-contamination.

4. Calculate the partition coefficient K_{av} of the standards.

K_{av} is expressed as:

$K_{av}=(V_e-V_0)/(V_c-V_0)$,

where V_e is the volume at which the peak was eluted, V_0 is the exclusion volume and V_c is the volume of the column. V_0 is the elution volume of the blue dextran.

Plot K_{av} against the logarithm of the molecular weight of the standards.

Calculate K_{av} of the sample peaks and deduce the molecular weight of the proteins and aggregates using the calibration curve.

5. An anticipated elution profile of, α-lactalbumin (α-la), β-lactoglobulin (β-lg) and heat-induced aggregates of whey proteins at 280 nm is shown in **Figure 3**.

(Figure 3 near here)

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4. Notes

1. TFA (trifluoroacetic acid) is an anionic ion-pairing agent interacting with the stationary phase of the column and with the positively charged portions of hydrophilic proteins and peptides, affecting their retention time. TFA is also UV-transparent, which makes it a suitable additive to HPLC solvents. TFA is very volatile; it is recommended to first add the acetonitrile or water and then the TFA when preparing the mobile phase to avoid loss. Due to its acute toxicity, it must be handled in the fume hood while wearing the appropriate PPE.
2. Buffers containing acetonitrile are very stable and no microbial growth is expected. Thus, the addition of sodium azide is not necessary and the mobile phase can be used for up to one year in an air-tight bottle.
3. The addition of 10% (v/v) water reduces the differences in viscosity of the two mobile phases (organic and aqueous) and improves mixing in the HPLC separation module before entering the column.
4. Mixing acetonitrile with water causes an endothermic reaction and a cooling of the mobile phase can be observed. Waiting for the solution to reach room temperature minimises error in the volume adjustment, thereby improving reproducibility.
5. The addition of TFA reduces the pH and also limits the risk of microbial growth. Thus, the aqueous mobile phase containing TFA can be used for several months after preparation; the addition of sodium azide is not necessary.
6. Sodium phosphate buffer containing 0.05% (w/v) sodium azide can be used for up to one month after preparation.
7. Sodium acetate/acetic acid buffer can be used within a few months due to the low pH of the buffer.
8. Guanidine hydrochloride (6 M) and dithiotreitol (19.5 mM) can be used as denaturing and reducing agent instead of urea and β-mercaptoethanol to improve the separation of some of the proteins **(*11,12*)**.

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9. The buffer should be freshly prepared and cannot be stored for long due to the high concentration of urea. The prolonged storage of urea leads to the formation of crystals.

10. In case of samples with a low protein concentration, a ratio sample: denaturing buffer of 1:4 can be used.

11. The adjustment of the protein concentration to around 0.25% (w/v) is an indicative figure. We observed a reasonable separation at this protein concentration, but this can be adjusted if necessary.

12. Urea denatures the proteins by disrupting hydrogen bonds. This requires a high concentration of urea. Without heating, β-mercaptoethanol requires more time to reduce the disulphide bonds of the proteins.

13. The choice of the adequate wavelength of detection can be made prior chromatographic separation by measuring an absorption spectrum of the sample with a UV/vis spectrophotometer. Most proteins and peptides contain aromatic amino acids that absorb at 280 nm. For some polypeptides, such as caseinomacropeptide, and generally shorter peptides, a detection wavelength of 214 nm is recommended, which corresponds to the absorption by the peptide bonds.

14. Centrifugation of the proteins at pH 4.6 allows the isoelectric precipitation of aggregated and denatured whey proteins. The centrifugation speed and duration are chosen to minimise the loss of native proteins in the pellet.

15. Using the original composition of the mobile phase for diluting the sample prevents a potential precipitation of the proteins in the column during the HPLC run. Precipitation of proteins in a chromatography column can cause irreversible blockages and damage to the stationary phase. Measuring the protein content before and after filtration is recommended to verify their solubility. Native proteins can be rehydrated in water unless precipitation is expected in the mobile phase.

16. C4 columns **(*13*)** or a PLRP-S column from Latek (Eppelheim, Germany) can also be used as an alternative **(*14*)**. The elution gradient and acetonitrile/water ratio of the mobile phases were, in both case, slightly modified.

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17. The use of two columns in series increases the number of theoretical plates and thus the quality of the separation. Using two G2000 columns of 300 mm or one G2000 column of 600 mm gives good results to analyse the disappearance of native whey proteins and appearance of soluble aggregated proteins during heat-treatment. The exclusion volume of the G3000 column corresponds to a higher molecular weight (5×10^5 Da) and allows the detection of larger aggregates. The OHpak SB-806 HQ-type column from Shodex (Tokyo, Japan), can separate even larger aggregates (exclusion volume corresponding to 2×10^7 Da). It is noteworthy that these SEC columns are very stable if treated with care.

18. The results obtained by SEC-HPLC are less sensitive to temperature variations because hydrophobic interactions with the stationnary phase are minimal, contrary to RP-HPLC.

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5. Acknowledgment

The authors would like to thank Bernard Corrigan for providing an example chromatogram of skim milk. This work was supported by the Dairy Levy Research Trust (project MDDT6261 “ProPart”), the Food Institutional Research Measure (FIRM, project no. 08RDTMFRC650) and Enterprise Ireland as part of the Food for Health Ireland Project (Grant Number CC20080001). S. J. Gaspard was funded under the Teagasc Walsh Fellowship Scheme (reference number 2012211).

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Figure captions

Figure 1: Anticipated results (method 1) of elution of κ-casein (κ-CN), α_{s2}-casein (α_{s2}-CN), α_{s1}-casein (α_{s1}-CN), β-casein (β-CN), α-lactalbumin (α-la), β lactoglobulin (β-lg A and B) from skim milk on a Poroshell 300SB-C18 column (Agilent, Santa Clara, CA, USA) at flow rate of 0.5 mL/min. The mobile phase A was 10% (v/v) acetonitrile (ACN) + 0.1% (v/v) trifluoroacetic acid (TFA) in water and the mobile phase B was 90% (v/v) ACN + 0.1% (v/v) TFA in water.

Figure 2: Anticipated chromatograms (method 2) of native caseinomacropeptide (CMP), α-lactalbumin (α-la) and β-lactoglobulin (β-lg A and B) on a C5 PolymerX RP1 column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min, detected at 280 nm (A) and 214 nm (B). The mobile phase A was 0.1% TFA (v/v) in water and the mobile phase B was 90% (v/v) ACN + 0.1% (v/v) TFA in water.

Figure 3: Chromatograms (method 3) showing the typical profile of (A) the whey proteins α-lactalbumin (α-la) and β-lactoglobulin (β-lg), and (B) heat-induced aggregates of α-lactalbumin and β-lactoglobulin on TSK Gel G2000SW_{XL} and TSK Gel G3000SW_{XL} in series (Tosoh Bioscience GmbH, Griesheim, Germany) eluted at a flow rate of 0.5 mL/min by SEC-HPLC. The mobile phase was 20 mM sodium phosphate (pH 7.0).

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362 **Table captions**

363 **Table 1**

364 Protein standards for SEC-HPLC of protein aggregates on a TSK Gel G2000SW_{XL} and a TSK Gel

365 G3000SW_{XL} in series (Tosoh Bioscience GmbH, Griesheim, Germany).

366 **Table 2**

367 Gradient of elution for the separation of caseins and whey proteins on a Poroshell 300SB-C18

368 column (Agilent, Santa Clara, CA, USA). Solvent A: 10% (v/v) ACN in 0.1% (v/v) TFA; solvent B:

369 90% (v/v) ACN in 0.1% (v/v) TFA.

370 **Table 3**

371 Gradient of elution for the separation of native whey proteins on a C5 PolymerX RP1 column

372 (Phenomenex, Torrance, CA, USA). Solvent A: 0.1% (v/v) TFA; solvent B: 90% (v/v) ACN in 0.1%

373 (v/v) TFA.

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375 **Table 1**

Protein	Molecular weight (Da)
Blue dextran	>2,000,000
Thyroglobulin	669,000
Ferritin	440,000
Aldolase	158,000
Bovine serum albumin	66,267
β-lactoglobulin	18,362
α-lactalbumin	14,174

376 Proteins can be purchased as a high molecular weight kit (GE Healthcare, Little Chalfont, U.K.) in
377 addition to bovine serum albumin, β-lactoglobulin and α-lactalbumin (Sigma Aldrich, St. Louis,
378 MO, USA).

379 **Table 2**

Time (min)	% A	% B
0.0	74	26
10.0	63	37
23.0	55	45
26.0	0	100
29.5	0	100
32.5	74	26
35.0	74	26

380

381 **Table 3**

Time (min)	% A	% B
0	80	20
3	80	20
13	60	40
33	40	60
35	0	100
40	0	100
40.5	80	20
45	80	20

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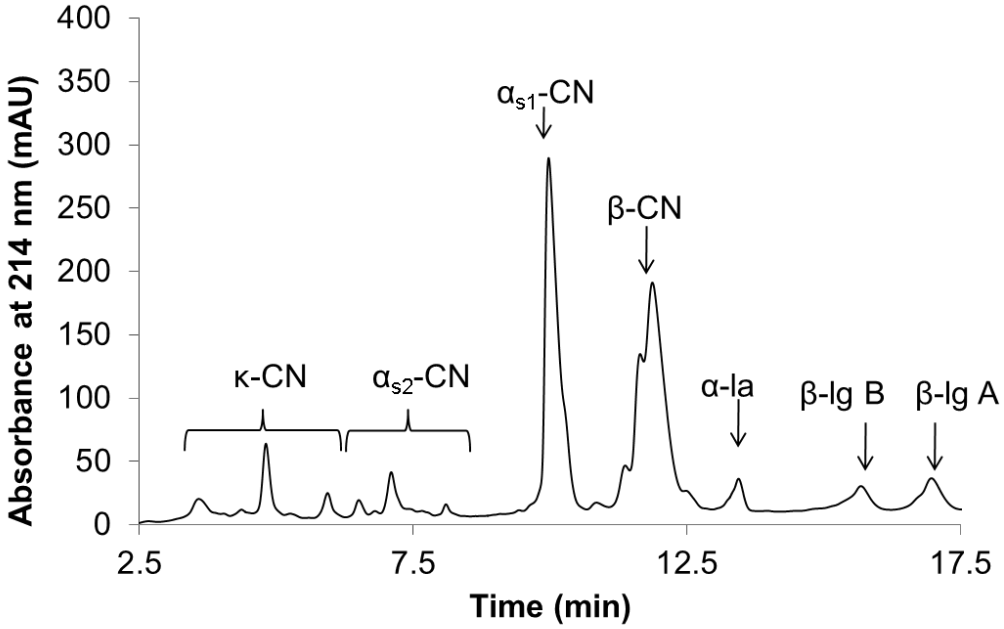

384

385 **Fig. 1**

386

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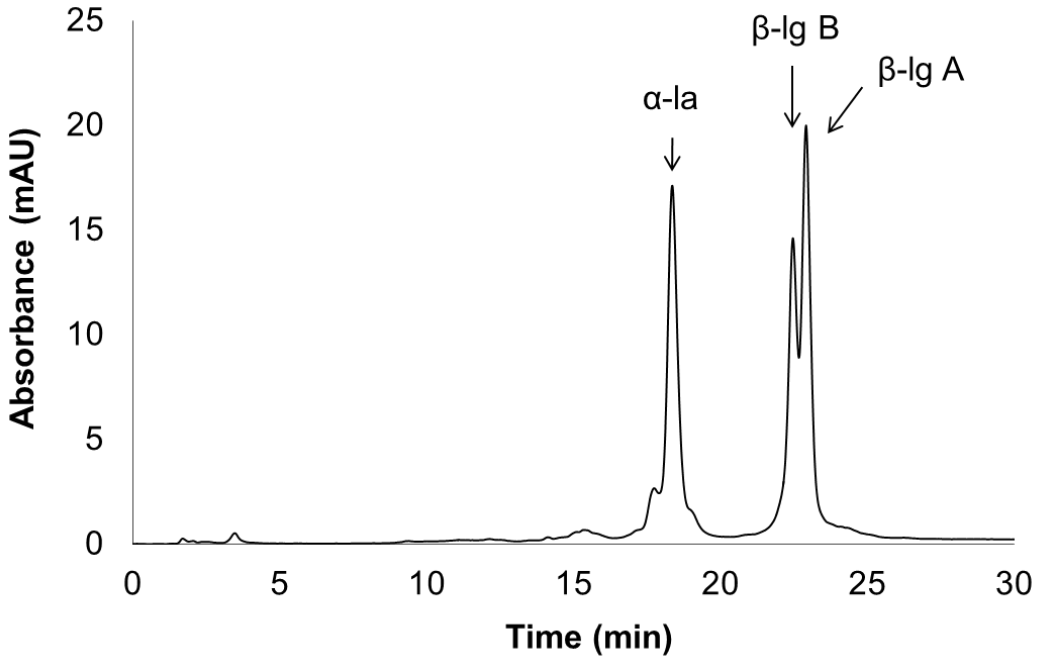
387 (A)



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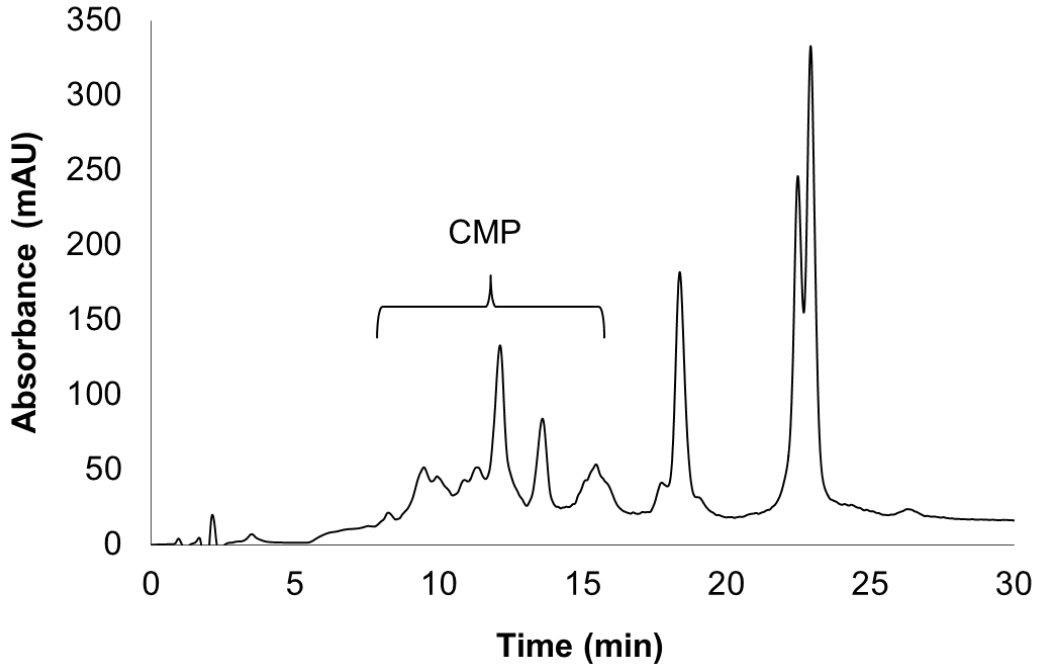
389 (B)



390

391 **Fig. 2**

392

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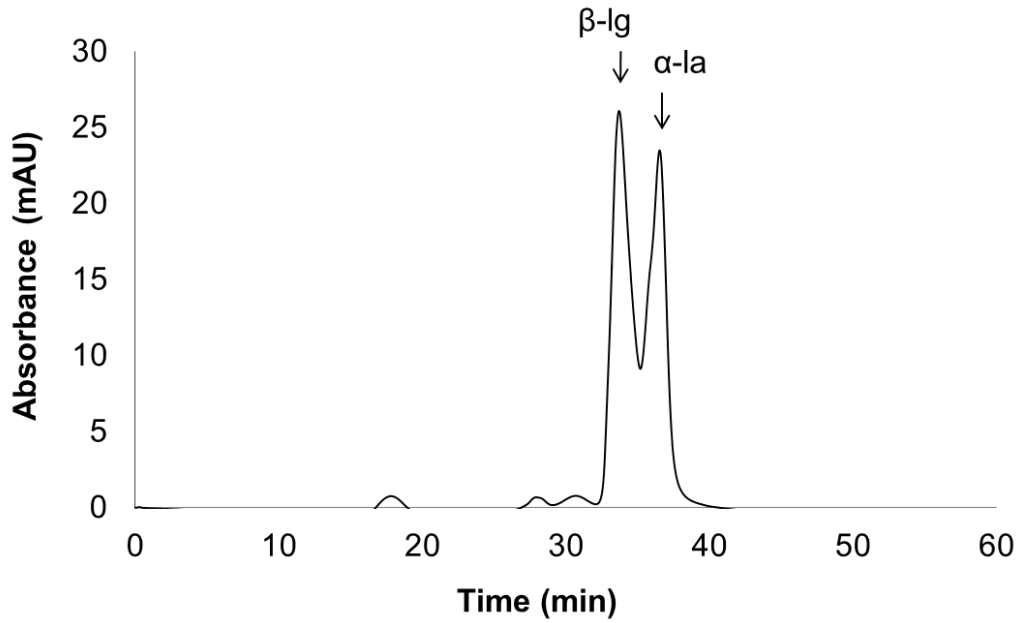
393 (A)



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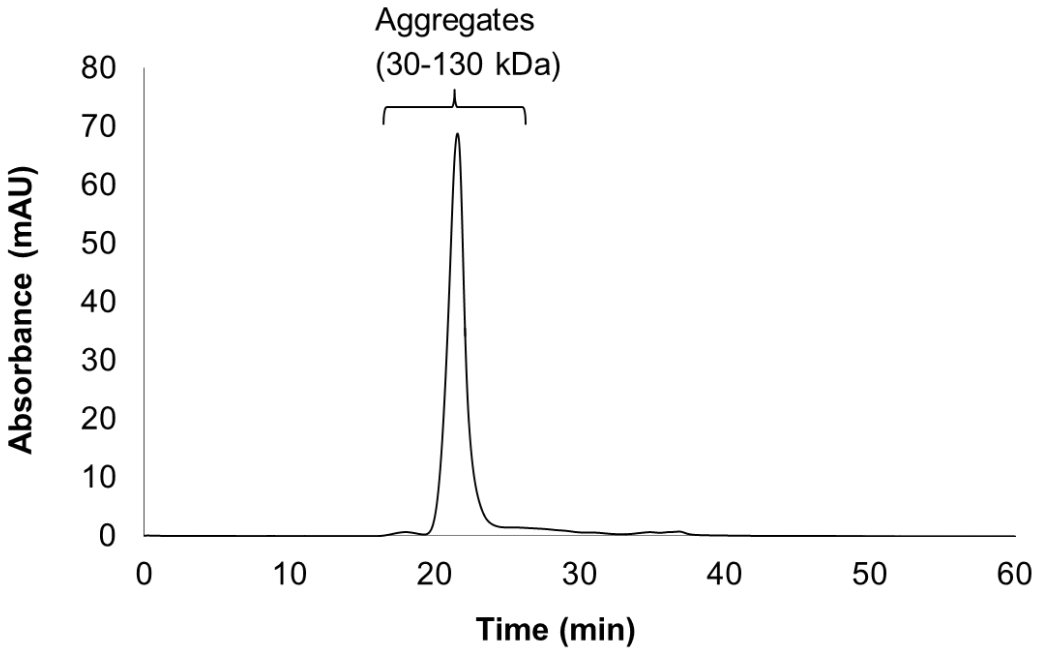
395 (B)



396

397 **Fig. 3**

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